

# Anti-Apolipoprotein A-1 auto-antibodies are active mediators of atherosclerotic plaque vulnerability

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## Aims

Anti-Apolipoprotein A-1 auto-antibodies (anti-ApoA-1 IgG) represent an emerging prognostic cardiovascular marker in patients with myocardial infarction or autoimmune diseases associated with high cardiovascular risk. The potential relationship between anti-ApoA-1 IgG and plaque vulnerability remains elusive. Thus, we aimed to investigate the role of anti-ApoA-1 IgG in plaque vulnerability.

## Methods and results

Potential relationship between anti-ApoA-1 IgG and features of cardiovascular vulnerability was explored both *in vivo* and *in vitro*. *In vivo*, we investigated anti-ApoA-1 IgG in patients with severe carotid stenosis ( $n = 102$ ) and in ApoE $-/-$  mice infused with polyclonal anti-ApoA-1 IgG. *In vitro*, anti-ApoA-1 IgG effects were assessed on human primary macrophages, monocytes, and neutrophils. Intr plaque collagen was decreased, while neutrophil and matrix metalloprotease (MMP)-9 content were increased in anti-ApoA-1 IgG-positive patients and anti-ApoA-1 IgG-treated mice when compared with corresponding controls. In mouse aortic roots (but not in abdominal aortas), treatment with anti-ApoA-1 IgG was associated with increased lesion size when compared with controls. In humans, serum anti-ApoA-1 IgG levels positively correlated with intraplaque macrophage, neutrophil, and MMP-9 content, and inversely with collagen. *In vitro*, anti-ApoA-1 IgG increased macrophage release of CCL2, CXCL8, and MMP-9, as well as neutrophil migration towards TNF- $\alpha$  or CXCL8.

## Conclusion

These results suggest that anti-ApoA-1 IgG might be associated with increased atherosclerotic plaque vulnerability in humans and mice.

## Keywords

Inflammation • Metalloproteases • Leucocytes • Carotid arteries

## Introduction

During the past decades, the concept of global 'cardiovascular vulnerability' opened new perspective in cardiovascular prevention.<sup>1,2</sup>

The possible identification of serum markers reflecting intraplaque vulnerability (easier to be measured than intraplaque parameters) could be of particular importance in both primary and secondary prevention of cardiovascular diseases. Simultaneously, it was

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established that atherosclerosis is not only a collection of cholesterol, complicated by smooth muscle cell proliferation, but also a chronic inflammatory disease, involving both innate and adaptive immunity. Cellular immunity is a key player in this process, but humoral immunity and autoantibody production also play an important role.<sup>3</sup> Some autoantibodies seem to be protective, but others are detrimental and associated with accelerated atherosclerosis and cardiovascular diseases.<sup>4</sup>

Among those, we have recently reported the presence of IgG autoantibodies directed against Apolipoprotein A-1 (anti-ApoA-1 IgG), the major fraction of high-density lipoprotein, as an independent predictor of major cardiovascular events, both in rheumatoid arthritis (RA) patients in primary prevention, and in myocardial infarction (MI) patients in secondary prevention.<sup>5,6</sup> High levels of anti-ApoA-1 IgG were also reported in patients with systemic lupus erythematosus and cardiovascular diseases.<sup>7</sup> In those clinical settings, anti-ApoA-1 IgG positivity was significantly associated with high serum levels of oxidised low-density lipoprotein and matrix metalloprotease (MMP)-9, two other possible systemic markers of atherosclerotic plaque vulnerability.<sup>5,8–11</sup>

Therefore, we investigated the potential involvement of anti-ApoA-1 IgG as a potential active factor in plaque vulnerability in humans, mice, and *in vitro* experiments. Patients without autoimmune or inflammatory diseases and asymptomatic for ischaemic stroke, which underwent carotid endarterectomy (CEA) for severe plaque stenosis, were tested for anti-ApoA-1 IgG. Positive and negative patients were compared for chemokine and MMP serum levels and plaque composition. To strengthen these observations, plaque composition was also assessed in ApoE<sup>−/−</sup> mice treated with goat polyclonal anti-ApoA-1 IgG. Finally, potential direct pro-atherosclerotic effects of anti-ApoA-1 IgG were tested in human primary neutrophil, monocyte, and macrophage functions.

## Methods

For additional details, please see the online Supplementary data.

### Patients and study design

We conducted a cohort study between March 2008 and April 2010 at a single hospital (San Martino Hospital) in Genoa (Italy). Patients ( $n = 102$ ), which underwent CEA for severe internal carotid stenosis incidentally diagnosed at US Doppler ( $>70\%$  luminal narrowing) and which did not present personal history of ischaemic cerebral symptoms, were enrolled in the study. Importantly, magnetic resonance imaging with diffusion sequences did not show any signs of cerebral necrosis in all patients enrolled. The degree of luminal narrowing was determined by repeated Doppler echography and angiographic confirmation using the criteria of the North American Symptomatic Carotid Endarterectomy Trial (NASCET).<sup>12</sup> The indication for CEA for asymptomatic patients was based on the recommendations published by the Asymptomatic Carotid Surgery Trial (ACST) and the indication for patients' symptomatic was according to the recommendations of the European Carotid Surgery Trial (ECST) and the NASCET.<sup>13–15</sup> The day before endarterectomy, blood samples were obtained by peripheral venipuncture from these patients at fasting state to collect serum and to perform blood parameters.

Medications reported in Table 1 were not modified in the 2 months prior to enrolment.

All patients who developed spontaneous cerebral embolism during 30 min preoperatively and during the dissection phase of the operation (detected by Transcranial Doppler insonation of the middle cerebral artery) were excluded from the study. Other exclusion criteria were malignant hypertension, acute coronary artery disease and unstable angina, any cardiac arrhythmias, congestive heart failure (II, III, and IV NYHA classes), liver or renal disorders or function abnormalities, acute and chronic infectious diseases, autoimmune and rheumatic diseases, rheumatoid factors, antinuclear antibody and anti-extractable nuclear antigen antibody serum positivity, cancer, endocrine diseases, inflammatory bowel diseases and anti-inflammatory (other than aspirin) medications, oral anticoagulant treatments, hormone, cytokine, or growth factor therapies.

The Medical Ethics Committee of San Martino Hospital approved the study, and participants provided written informed consent. The study was conducted in compliance with the Declaration of Helsinki.

### Animals

Eleven-week-old ApoE<sup>−/−</sup> C57BL/6 mice were submitted to passive immunization treatment protocol as described in detail in online Supplementary data.

### Determination of human autoantibodies anti-ApoA-1 by ELISA

Anti-ApoA-1 IgG were measured as previously described.<sup>5,6</sup> This method was performed as described in the online Supplementary data.

### Detection of inflammatory mediators in human serum and cell supernatants

This method was performed as described in the online Supplementary data.

### Pro-matrix metalloprotease-9 zymographic assay

This method was performed as described in the online Supplementary data.

### Human carotid plaque specimen processing

This method was performed as described in the online Supplementary data.

### Immunohistochemistry in human carotid plaques and mouse aortic sinus

This method was performed as described in detail in the online Supplementary data.

### Oil Red O staining for lipid content

This method was performed as described in detail in the online Supplementary data.

### Sirius Red staining for collagen content

This method was performed as described in detail in the online Supplementary data.

### Real-time RT-PCR

This method was performed as described in detail in the online Supplementary data.

**Table 1** Clinical characteristics and medications of study population

Characteristics	Anti-ApoA-1 IgG negative (n = 82)	Anti-ApoA-1 IgG positive (n = 20)	P-value
Age, year (IQR)	72 (67–76)	71.5 (66.5–81)	0.10
Males, n (%)	53 (65)	13 (65)	1.00
Systolic blood pressure, mmHg (IQR)	140 (130–150)	145 (130–150)	0.56
Diastolic blood pressure, mmHg (IQR)	80 (80–85)	80 (80–81)	0.92
Waist circumference, cm (IQR)	93.5	86	0.29
Current smoking, n (%)	21 (26)	10 (50)	0.06
Type 2 diabetes, n (%)	13 (16)	2 (10)	0.73
Dyslipidaemia, n (%)	49 (60)	15 (75)	0.30
Hypertension, n (%)	59 (72)	15 (75)	1.00
Chronic CAD, n (%)	14 (17)	6 (30)	0.21
Total WBC, $n \times 10^9/L$ (IQR)	7.1 (6.2–8.1)	8.0 (6.2–9.7)	0.18
Neutrophils, $n \times 10^9/L$ (IQR)	4.5 (3.5–5.2)	5.3 (4–6.8)	0.08
Lymphocytes, $n \times 10^9/L$ (IQR)	1.77 (1.44–2.14)	1.86 (1.18–2.10)	0.60
Monocytes, $n \times 10^9/L$ (IQR)	0.44 (0.37–0.58)	0.46 (0.39–0.60)	0.42
Red blood cells, $n \times 10^{12}/L$ (IQR)	4.7 (4.4–5)	4.6 (4.35–4.90)	0.73
Platelet, $n \times 10^9/L$ (IQR)	244 (210–297)	250 (187.5–305)	0.95
Plasma fibrinogen, g/L (IQR)	3.62 (3.14–4.10)	3.73 (3.04–4.53)	0.38
Serum ApoA-1, mg/dL (IQR)	167 (122–217)	208.5 (165–222)	0.08
Serum total-c, mg/dL (IQR)	199 (172–231)	172 (158–198)	0.01
Serum LDL-c, mg/dL (IQR)	117 (90.8–149)	106.5 (88–126)	0.1
Serum HDL-c, mg/dL (IQR)	48 (40–60)	47.5 (42.5–55)	0.88
Serum triglycerides, mg/dL (IQR)	127 (92–175)	102.5 (81.5–138)	0.01
Serum glycaemia, mg/dL (IQR)	98 (89–112)	101 (92–112)	0.59
Serum insulinaemia, mU/L (IQR)	9.5 (6.2–11.6)	8.8 (5.5–17.5)	0.88
Anti-platelets, n (%) aspirin	46 (56)	15 (75)	0.79
Clopidogrel	16 (20)	3 (15)	0.76
Diuretics, n (%)	4 (5)	3 (15)	0.13
ACE inhibitors, n (%)	2 (2)	1 (5)	0.48
ARBs, n (%)	37 (45)	10 (50)	0.89
Beta-blockers, n (%)	24 (29)	5 (25)	0.79
Calcium channel blockers, n (%)	23 (28)	8 (40)	0.42
Statins, n (%)	37 (45)	12 (60)	0.32
Oral anti-diabetics, n (%)	8 (10)	2 (10)	1.00
% Carotid lumen stenosis (IQR)	80 (75–90)	75 (70–80)	0.30

Continuous variables are expressed as median [interquartile range (IQR)].

CAD, coronary artery disease; WBC, white blood cells; total-c, total cholesterol; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; ACE, angiotensin converting enzyme; ARBs, angiotensin receptor blockers.

## Human primary neutrophil isolation and migration assay

This method was performed as described in detail in the online Supplementary data.

## Human monocyte isolation and migration assay

This method was performed as described in detail in the online Supplementary data.

## Human primary macrophage differentiation and culture

This method was performed as described in detail in the online Supplementary data.

## Statistical analysis

Patient characteristics were described 1 day before endarterectomy. Anti-ApoA-1 IgG-positive patients were compared with negative patients using Pearson's Chi-square test or Fisher's exact test, when appropriate, for the comparison of qualitative variables and Mann–Whitney non-parametric test (the normality assumption of the variables' distribution in both groups was violated) for comparisons of continuous variables. The comparisons between upstream and downstream portions of carotid plaques within anti-ApoA-1 IgG-positive and -negative groups were performed using Mann–Whitney *U*-test. Comparisons between parameters of mouse plaque vulnerability in anti-ApoA-1 IgG-, isotype control IgG-, and vehicle (PBS)-treated mice were performed using Mann–Whitney *U*-test. For continuous variables, results were expressed as medians [interquartile range (IQR)].

Spearman's rank correlation coefficients were used to assess correlations between anti-ApoA-1 IgG serum levels and, respectively, intra-plaque infiltration of vascular and inflammatory cells, collagen and MMP-9 content, or inflammatory gene mRNA expression ( $\Delta$ CT) in both upstream and downstream regions of carotid atherosclerotic plaques.

*In vitro* results were expressed as mean ( $\pm$  SD) (neutrophil and monocyte chemotaxis assays) and as medians (IQR) (macrophage cultures). One-way ANOVA was used for multiple group comparison, while unpaired *t*-test for two-group comparison. Values of  $P < 0.05$  (two-tailed) were considered significant. All analyses were done with Statistica™ software (StatSoft, Tulsa, OK, USA) and Analyse-it® (Analyse-it Software, Leeds, UK) software.

## Results

### Patient characteristics

Clinical and demographic characteristics, biological parameters as well as medications in patients with severe internal carotid stenosis and asymptomatic for ischaemic stroke are described in Table 1. Serum anti-ApoA-1 IgG were positive in 20 (19.6%) patients, which are similar to what have been previously observed in patients with acute coronary syndrome.<sup>6,8</sup> There was no significant difference between patients negative or positive for anti-ApoA-1 IgG in terms of age, sex, and medications. Although most cardiovascular risk factors were not different between the two groups, total serum cholesterol and triglycerides were increased in anti-ApoA-1 IgG-negative patients (respectively, total cholesterol: 199 vs. 172 mg/dL,  $P = 0.01$  and triglycerides 127 vs. 102.5 mg/dL,  $P = 0.01$ ).

### Systemic levels of inflammatory biomarkers

No significant differences in serum levels for C-reactive protein, TNF- $\alpha$ , CCL2, CCL3, and MMP-8 were observed between anti-ApoA-1 IgG-negative and -positive patients. Although a slight increase in CCL4 and MMP-9 levels was observed in patients positive for anti-ApoA-1, differences were not statistically significant (respectively, CCL4:  $P = 0.08$ ; MMP-9:  $P = 0.06$ , Table 2). Nevertheless, increased gelatinolytic activity for serum pro-MMP-9 was shown in anti-ApoA-1 IgG positive when compared with negative patients (Table 2).

### Positive serum levels of anti-ApoA-1 IgG are associated with the increase of atherosclerotic plaque vulnerability in humans

We then looked for a difference in plaque vulnerability between patients positive and negative for serum anti-Apo A-1 IgG. In upstream portions of carotid plaques, collagen III content was decreased in anti-ApoA-1 IgG positive when compared with negative patients (Table 3). Accordingly, intraplaque macrophage and MMP-9 content were significantly increased in anti-ApoA-1 IgG positive when compared with negative patients. A slight increase in MMP-8 mRNA expression was also observed in positive when compared with negative patients, but the difference was not

**Table 2** Systemic cardiovascular risk markers

Cardiovascular markers	Anti-ApoA-1 negative (n = 82)	Anti-ApoA-1 positive (n = 20)	P-value
Hs C-reactive protein, mg/L	1.8 (0.8–5.0)	2.4 (1–3.4)	0.73
TNF- $\alpha$ , pg/mL	15.6 (15.6–19.8)	15.6 (15.6–23.9)	0.47
CCL2, pg/mL	15.6 (15.6–65.3)	27.05 (15.6–151.5)	0.30
CCL3, pg/mL	8.4 (7.7–13.3)	8.7 (7.8–31.9)	0.84
CCL4, pg/mL	30.0 (17.3–52.6)	37.0 (31.3–67.2)	0.08
MMP-9, ng/mL	300 (73–615)	549 (218–1027)	0.06
Pro-MMP-9 activity	20.1 (15.1–28.4)	30.2 (23.8–38.7)	0.009
MMP-8, ng/mL	7.3 (1.3–15.5)	10.4 (6–17.5)	0.15

Data are expressed as median [interquartile range (IQR)].

Hs C-reactive protein, high-sensitivity C-reactive protein; TNF, tumour necrosis factor; MMP, matrix metalloprotease.

statistically significant ( $P = 0.08$ ). Similar to upstream regions, in downstream portions of carotid plaques, collagen content was decreased in anti-ApoA-1 IgG positive when compared with negative patients. Accordingly, macrophage and MMP-9 content were increased in anti-ApoA-1 IgG-positive patients. Different from upstream, in downstream portions, neutrophil intraplaque infiltration was markedly increased in anti-ApoA-1 IgG positive when compared with negative patients. No significant differences were observed in other intraplaque parameters, such as lipid, smooth muscle cell and lymphocyte content, or inflammatory gene mRNA expression (Table 3). Circulating anti-ApoA-1 IgG levels inversely correlated with collagen content (upstream collagen III:  $r = -0.35$ ,  $P = 0.001$ ; downstream total collagen:  $r = -0.29$ ,  $P = 0.008$ ), while positively correlated with macrophage, neutrophil, and MMP-9 content in both upstream and downstream portions of carotid plaques (upstream: macrophages:  $r = 0.28$ ,  $P = 0.009$ ; neutrophils:  $r = 0.31$ ,  $P = 0.004$ ; MMP-9:  $r = 0.40$ ,  $P = 0.001$ ; downstream: macrophages:  $r = 0.33$ ,  $P = 0.002$ ; neutrophils:  $r = 0.43$ ,  $P = 0.0001$ ; MMP-9:  $r = 0.42$ ,  $P = 0.0001$ ) (Table 4). Importantly, no significant correlations were observed between anti-ApoA-1 serum levels and other intraplaque parameters (Table 4). Taken together, these results indicate that atherosclerotic plaque from patients positive for serum anti-Apo A-1 IgG are more vulnerable than those from anti-Apo A-1 IgG-negative patients.

### Anti-ApoA-1 IgG treatment increases intraplaque vulnerability in ApoE $^{-/-}$ mice

To confirm these observations, we determined the impact of anti-ApoA-1 IgG levels on mouse plaque vulnerability parameters. We intravenously injected 11-week-old ApoE $^{-/-}$  mice with goat polyclonal anti-human ApoA-1 IgG, respective goat IgG controls, or vehicle (PBS), every 2 weeks over 16 weeks. Mice were fed before and during treatments with standard chow diet to avoid the induction of severe hypercholesterolaemia and render lipid

**Table 3** Parameters of intraplaque vulnerability

Carotid intraplaque parameters	Anti-ApoA-1 negative (n = 82)	Anti-ApoA-1 positive (n = 20)	P-value
Plaque size, cm	1.7 (1.5–2)	1.5 (1.3–1.9)	0.38
Plaque stenosis, % of lumen	80 (75–90)	75 (70–80)	0.30
Upstream portion			
% lipid	5.98 (2.86–9.37)	7.11 (3.31–10.81)	0.42
% total collagen	33.58 (26.15–40.06)	33.29 (25.21–39.24)	0.88
% collagen I	11.54 (9.44–16.9)	13.36 (7.69–15.05)	0.25
% collagen III	13.17 (10.03–17.27)	9.82 (7.55–12.42)	0.005
% of smooth muscle cell-rich area	5.39 (3.16–9.37)	3.53 (2.33–5.85)	0.09
% of macrophage-rich area	4.98 (2.73–9.94)	7.95 (5.59–13.95)	0.02
Lymphocytes/mm <sup>2</sup>	2.37 (1.12–5.93)	4.03 (1.62–13.67)	0.14
Neutrophils/mm <sup>2</sup>	2.35 (1.12–6.01)	3.27 (2.18–8.44)	0.19
MMP-8 mRNA, fold increase	1.23 (0.38–3.00)	2.56 (0.66–3.86)	0.08
% MMP-9	2.99 (1.29–5.87)	6.25 (2.40–12.6)	0.02
TNF- $\alpha$ mRNA, fold increase	1.20 (0.54–1.95)	1.22 (0.49–1.94)	0.72
CCL2 mRNA, fold increase	0.93 (0.64–1.39)	1.17 (0.65–1.67)	0.37
CCL3 mRNA, fold increase	0.91 (0.62–1.72)	1.15 (0.79–1.58)	0.71
CCL4 mRNA, fold increase	0.88 (0.55–1.53)	1.02 (0.51–1.31)	0.65
CXCL8 mRNA, fold increase	0.96 (0.52–1.87)	1.32 (0.82–2.00)	0.39
Downstream portion			
% lipid	3.45 (1.64–8.37)	4.78 (1.36–10.05)	0.57
% total collagen	20.88 (17.20–23.64)	17.90 (12.82–20.49)	0.01
% collagen I	6.40 (4.27–11.29)	5.45 (2.98–7.04)	0.06
% collagen III	6.63 (4.11–10.28)	4.58 (3.27–5.84)	0.03
% of smooth muscle cell-rich area	2.87 (1.62–4.38)	2.69 (1.69–3.88)	0.69
% of macrophage-rich area	6.88 (2.62–16.12)	11.17 (7.44–18.37)	0.04
Lymphocytes/mm <sup>2</sup>	2.09 (0.98–5.37)	2.81 (1.24–11.50)	0.34
Neutrophils/mm <sup>2</sup>	2.98 (0.66–8.51)	9.85 (3.88–15.12)	0.002
MMP-8 mRNA, fold increase	1.13 (0.34–3.25)	1.19 (0.80–2.28)	0.95
% MMP-9	4.77 (1.94–9.66)	19.37 (6.76–25.69)	<0.0001
TNF- $\alpha$ mRNA, fold increase	1.46 (0.78–2.77)	0.96 (0.62–2.06)	0.08
CCL2 mRNA, fold increase	1.37 (0.78–2.05)	1.95 (1.15–2.38)	0.07
CCL3 mRNA, fold increase	1.37 (0.84–2.14)	1.63 (0.58–2.18)	0.87
CCL4 mRNA, fold increase	1.38 (0.89–2.35)	1.75 (0.64–2.45)	0.91
CXCL8 mRNA, fold increase	1.16 (0.54–2.28)	0.91 (0.41–1.71)	0.32

Data are expressed as median [interquartile range (IQR)].  
MMP, matrix metalloprotease.

levels more similar to that detected in human patients.<sup>16</sup> At sacrifice, the ratio of serum cholesterol sub-fractions and triglycerides was similar in three mouse groups (Supplementary material online, Table S1). Atherosclerotic lesion size in thoracoabdominal aortas was comparable in all groups (lipid deposition on total aorta surface: PBS:  $2.5 \pm 0.8\%$ ; CTL IgG:  $3.6 \pm 1.3\%$ ; anti-ApoA-1:  $3.4 \pm 0.9\%$ ). In aortic roots, atherosclerotic lesion size (as determined by Oil Red O staining) was increased in anti-ApoA-1 IgG-treated mice when compared with PBS or CTL IgG treatments (Table 5, Supplementary material online, Figures S1 and S2). Neutrophil, MMP-8, and MMP-9 contents were also increased in anti-ApoA-1 IgG-treated mice when compared with control groups (Table 5, Supplementary material online, Figures S1 and S2). Accordingly, total collagen content was significantly

reduced in anti-ApoA-1 IgG-treated mice when compared with PBS or CTL IgG treatments (Table 5, Supplementary material online, Figure S1). Despite a slight increase in intraplaque macrophage and lymphocyte infiltration in anti-ApoA-1 IgG-treated mice, no significant changes were observed between groups (Table 5, Supplementary material online, Figure S2). The analysis of mRNA expression of mediators of vulnerability [such as macrophage (Cd68), neutrophil (neutrophil elastase, Elane), and Mmp9] and T helper (Th) lymphocyte polarization in mouse abdominal aortas, spleen, and lymphnodes partially confirmed several findings retrieved on histology data (Supplementary material online, Table S4). These results indicate that anti-ApoA-1 IgG treatment was associated with increased plaque vulnerability parameters (i.e. neutrophils and MMPs).



**Table 4** Spearman rank correlation between anti-ApoA-1 IgG levels and human intraplaque lipids, collagen content, cell infiltration, MMP-9, and cytokine expression in the upstream or downstream regions

	Spearman's correlation coefficient (r)	P-value
Intraplaque upstream		
vs. lipids	0.06	0.562
vs. total collagen	−0.12	0.219
vs. collagen I	−0.21	0.048
vs. collagen III	−0.35	0.001
vs. SMC	−0.25	0.017
vs. macrophages	0.28	0.009
vs. lymphocytes	0.11	0.487
vs. neutrophils	0.31	0.004
vs. MMP-9	0.40	0.001
TNF- $\alpha$ mRNA, $\Delta$ CT	−0.03	0.820
CCL2 mRNA, $\Delta$ CT	−0.07	0.526
CCL3 mRNA, $\Delta$ CT	−0.10	0.447
CCL4 mRNA, $\Delta$ CT	−0.06	0.586
CXCL8 mRNA, $\Delta$ CT	−0.06	0.580
Intraplaque downstream		
vs. lipids	0.17	0.191
vs. total collagen	−0.29	0.008
vs. collagen I	−0.15	0.232
vs. collagen III	−0.14	0.245
vs. SMC	−0.04	0.725
vs. macrophages	0.33	0.002
vs. lymphocytes	0.13	0.334
vs. neutrophils	0.43	0.0001
vs. MMP-9	0.42	0.0001
TNF- $\alpha$ mRNA, $\Delta$ CT	0.06	0.557
CCL2 mRNA, $\Delta$ CT	−0.17	0.194
CCL3 mRNA, $\Delta$ CT	−0.13	0.337
CCL4 mRNA, $\Delta$ CT	0.10	0.469
CXCL8 mRNA, $\Delta$ CT	−0.04	0.755

SMC, smooth muscle cells.

### Treatment with anti-ApoA-1 IgG increases human neutrophil migration towards intraplaque chemoattractants

To investigate whether anti-ApoA-1 IgG could be responsible for neutrophil influx, we tested the effect of anti-ApoA-1 IgG on *in vitro* human neutrophil migration. Pre-incubation with goat polyclonal anti-ApoA-1 IgG (at 40  $\mu$ g/mL) significantly increased neutrophil migration towards CXCL8 and TNF- $\alpha$  (both expressed within atherosclerotic plaques) when compared with CTL medium or CTL IgG treatments (Table 6). On the contrary, short-term pre-treatment and co-incubation with goat polyclonal anti-ApoA-1 IgG (20–40  $\mu$ g/mL) during chemotaxis assays did not significantly alter primary human monocyte migration

towards classical chemoattractants (CCL2, CCL3, C-reactive protein) (Table 7).

### Treatment with anti-ApoA-1 IgG induces the release of CCL2, CXCL8, TNF- $\alpha$ , and matrix metalloproteinase-9 in human macrophage supernatants

To determine whether anti-ApoA-1 IgG could modulate the production of leucocyte chemoattractants and plaque vulnerability factors, we tested the effect of goat polyclonal anti-ApoA-1 IgG and respective control on human monocyte-derived macrophages *in vitro*. Moreover, to verify that human anti-ApoA-1 IgG had the same effects as goat anti-ApoA-1 IgG, we purified IgG from serum of anti-ApoA-1 IgG-positive and -negative patients with severe carotid stenosis and also tested them on monocyte-derived macrophages. Goat anti-ApoA-1 IgG significantly increased CCL2, CXCL8, and TNF- $\alpha$  release when compared with CTL medium or CTL IgG treatments (Table 8). Similarly, IgG from anti-ApoA-1-positive patients induced CCL2, CXCL8, and TNF- $\alpha$  production when compared with treatment with IgG isolated from negative patients (Table 8). Anti-ApoA-1 IgG significantly increased release of MMP-9 and gelatinolytic activity of pro-MMP-9 when compared with treatments with CTL IgG or IgG from negative patients (Table 8). No effect of anti-ApoA-1 IgG was observed on MMP-8 secretion in macrophage supernatants at 48 h of incubation (Table 8). The absence of LPS contamination of human isolated IgG, commercial anti-ApoA-1 IgG, and CTL IgG preparations was confirmed by Limulus assay (0.1 EU/mL for both antibodies, data not shown).

## Discussion

In this study, we demonstrated a positive association between serum anti-ApoA-1 IgG levels and features of atherosclerotic intraplaque vulnerability, such as an increased phagocyte (macrophages and neutrophils) and MMP-9 content, and a reduced intraplaque content of collagen in patients with severe but asymptomatic carotid stenosis. Moreover, most of the associations between anti-ApoA-1 IgG positivity and vulnerable atherosclerotic plaque features retrieved in humans (with the exception of intraplaque macrophages content) were reproduced and confirmed in ApoE  $-/-$  mice exposed to passive immunization with anti-ApoA-1 IgG. Indeed, even if mice exposed to anti-ApoA-1 IgG did not develop bigger atherosclerotic lesions, those lesions contained higher MMP-9, neutrophil content, and lower total collagen amount, which fulfil some of the possible characteristics of a prone-to-rupture atherosclerotic lesion.<sup>17</sup> In order to explore the potential mechanisms underlying these observational data in humans and mice, we investigated *in vitro* the role of anti-ApoA-1 IgG on both local and systemic processes regulating plaque vulnerability.

Local inflammation was considered by investigating anti-ApoA-1 IgG effects on human monocyte-derived macrophages, which are the most abundant inflammatory cell population in atherosclerotic lesions and have a key role in atherogenesis and plaque vulnerability.<sup>18</sup> When exposed to human macrophages, goat polyclonal

**Table 5** Parameters of mouse plaque vulnerability

Parameters	Vehicle-treated mice (n = 10)	CTL IgG-treated mice (n = 14)	Anti-ApoA-1 IgG-treated mice (n = 9)
Oil Red O, $\times 10^3 \mu\text{m}^2$	198 (171–216)	216 (185–232)	250 (227–297) <sup>†</sup>
Total collagen, %	41 (32–43)	40 (35–43)	35 (32–40) <sup>‡</sup>
Macrophage+ area, %	9 (6–11)	10 (6–16)	14 (10–16) <sup>§</sup>
Lymphocytes/mm <sup>2</sup>	48.3 (39.5–52.4)	47.1 (29.8–64.3)	49.7 (45.5–59.5) <sup>§</sup>
Neutrophils/mm <sup>2</sup>	5 (2–15)	14 (5–19)	20 (14–23) <sup>  </sup>
MMP-8, %	2 (2–4)	2 (2–4)	8 (4–9) <sup>#</sup>
MMP-9, %	10 (9–12)	11 (8–16)	21 (16–25) <sup>**</sup>

Data are expressed as median [interquartile range (IQR)].

MMP, matrix metalloproteinase.

<sup>†</sup>P < 0.01 vs. CTL IgG-treated mice.

<sup>‡</sup>P < 0.05 vs. CTL IgG-treated mice.

<sup>§</sup>P = NS (not significant).

<sup>||</sup>P < 0.05 vs. CTL IgG-treated mice.

<sup>#</sup>P < 0.05 vs. CTL IgG-treated mice.

<sup>\*\*</sup>P < 0.01 vs. CTL IgG-treated mice.

**Table 6** Control (CTL) isotype IgG- and goat anti-ApoA-1 IgG-treated human primary neutrophil migration in response to classical chemoattractants

Migration assay [polycarbonate, chemotaxis index (C.I.)]					
Human neutrophils					
Lower well (chemoattractant)	Upper well (treated cells)				
	CTL medium	CTL IgG (20 $\mu\text{g/mL}$ )	CTL IgG (40 $\mu\text{g/mL}$ )	Anti-apoA-1 IgG (20 $\mu\text{g/mL}$ )	Anti-ApoA-1 IgG (40 $\mu\text{g/mL}$ )
CTL medium	1.0 $\pm$ 0.0	1.32 $\pm$ 0.30	1.14 $\pm$ 0.23	0.99 $\pm$ 0.19	1.06 $\pm$ 0.16
CXCL8 (10 nM)	2.49 $\pm$ 0.49*	2.36 $\pm$ 0.12	2.28 $\pm$ 0.34	2.87 $\pm$ 0.98	3.15 $\pm$ 0.38 <sup>†</sup>
TNF- $\alpha$ (200 U/mL)	2.42 $\pm$ 0.38*	2.06 $\pm$ 0.12	2.20 $\pm$ 0.58	2.66 $\pm$ 0.68	3.1 $\pm$ 0.44 <sup>‡</sup>

Data are expressed as mean  $\pm$  SD (n = 5).

\*P < 0.05 vs. CTL medium-treated neutrophil migration towards CTL medium.

<sup>†</sup>P < 0.01 vs. CTL medium-treated neutrophil migration towards CXCL8.

<sup>‡</sup>P < 0.05 vs. CTL medium-treated neutrophil migration towards TNF- $\alpha$ .

anti-ApoA-1 IgG and purified IgG fraction from anti-ApoA-1 IgG-positive patients (preparations devoid of any other common auto-antibodies) elicited a pro-inflammatory response in terms of MMP-9 (both activity and protein concentration), CCL2, CXCL8, and TNF- $\alpha$ . These cytokines play a central role in the recruitment of inflammatory cells within atherosclerotic plaques.<sup>18,19</sup> On the other hand, MMP-9 selectively degrades gelatin, collagen type IV and V, thereby weakening the fibrous cap making plaques more prone to rupture.<sup>20</sup> Therefore, anti-ApoA-1 IgG could be directly involved in plaque vulnerability by promoting the production of pro-atherosclerotic factors.

Concerning the potential vulnerable activity of serum anti-ApoA-1 IgG at systemic levels (in the blood stream), we explored circulating monocyte and neutrophil migration in response to well-known chemoattractants expressed within atherosclerotic plaques. As shown by chemotaxis migration assays, anti-ApoA-1 IgG exposure increased neutrophil migration

towards CXCL8 and TNF- $\alpha$ , but not monocyte migration, suggesting that anti-ApoA-1 IgG selectively increased circulating neutrophil locomotion. This effect was partially due to direct chemokinetic properties of anti-ApoA-1 IgG, as suggested by the checkerboard analysis performed in the presence of different concentrations of anti-ApoA-1 IgG (data not shown). As neutrophils have been recently shown to play a crucial role in atherosclerotic plaque vulnerability, this observation supports the role of anti-ApoA-1 IgG in this process.<sup>19,21</sup>

However, the direct effect of anti-ApoA-1 IgG on neutrophil migration represents a surprising result, for which the mechanism is still unexplained. Other auto-antibodies, namely anti-neutrophil cytoplasmic antibodies, have been shown to promote migration of flowing neutrophils through endothelial cells, presumably by activating  $\beta_2$ -integrin-dependant immobilization.<sup>22</sup> On the other hand, in the human and mouse models of atherosclerosis, the positive association between anti-ApoA-1 IgG levels and the

**Table 7** Control (CTL) isotype IgG- and goat anti-ApoA-1 IgG-treated human primary monocyte migration in response to classical chemoattractants

Migration assay [polycarbonate, chemotaxis index (C.I.)]					
Lower well (chemoattractant)	Upper well (treated cells)				
	CTL medium	CTL IgG (20 µg/mL)	CTL IgG (40 µg/mL)	Anti-ApoA-1 IgG (20 µg/mL)	Anti-ApoA-1 IgG (40 µg/mL)
CTL medium	1.0 ± 0.0	0.92 ± 0.06	0.98 ± 0.21	1.01 ± 0.08	0.94 ± 0.22
CCL2 (10 nM)	2.19 ± 0.38	2.19 ± 0.52	2.26 ± 0.18	2.52 ± 0.45	2.62 ± 0.41
CCL3 (10 nM)	2.08 ± 0.38	2.19 ± 0.50	2.17 ± 0.39	2.60 ± 0.76	2.62 ± 0.41
C-reactive protein (40 µg/mL)	2.13 ± 0.56	2.34 ± 0.62	2.11 ± 0.25	2.39 ± 0.42	2.46 ± 0.26

Data are expressed as mean ± SD (n = 6). No comparisons between groups are statistically significant.

**Table 8** Secretion of neutrophil and monocyte chemoattractants, as well as MMP-8 and MMP-9 from human primary macrophages

Cell culture assay						
Mediator released	Stimulation					
	CTL medium	LPS (1 ng/mL)	CTL IgG (40 µg/mL)	Anti-ApoA-1 IgG (40 µg/mL)	anti-ApoA-1 IgG-negative patients	anti-ApoA-1 IgG-positive patients
CCL2, pg/mL	7 (5–11)	260* (233–2876)	6.9 (4.6–9.5)	291 <sup>†</sup> (225–1047)	10.8 (8–42.9)	173.7 <sup>§</sup> (134.7–264.7)
CXCL8, pg/mL	131 (62–180)	7158* (6591–16840)	51 (73–228)	4189 <sup>†</sup> (4181–11654)	209 (160–313)	1327 <sup>§</sup> (788–2274)
TNF-α, pg/mL	BDL	320* (225–456)	BDL	74 <sup>†</sup> (25–106)	BDL	33 <sup>§</sup> (12.1–52)
MMP-8, pg/mL	95 (57–168)	326* (54–3635)	1 (1–77)	14.8 <sup>‡</sup> (1–173)	71 (34–3192)	139 <sup>  </sup> (43–28359)
MMP-9, ng/mL	4.2 (0.4–62)	26* (14–32)	8.7 (4–13)	59 <sup>†</sup> (45–83)	8.4 (3.7–15.3)	40 <sup>§</sup> (16.6–59)
Pro-MMP-9 activity, ng/mL	16 (14–18)	50* (37–61)	21 (16–23)	32 <sup>‡</sup> (27–42)	18 (16–21)	33 <sup>§</sup> (25–40)

Data are expressed as median and (IQR) (n = 7). BDL, below the detection limit of the assay. For statistical analysis, the lower detection limit value was used.

\*P < 0.05 vs. control medium.

<sup>†</sup>P < 0.05 vs. control IgG.

<sup>‡</sup>P: NS (not significant) vs. control IgG.

<sup>§</sup>P < 0.005 vs. anti-ApoA-1 IgG-negative patients.

<sup>||</sup>P = NS (not significant) vs. anti-ApoA-1 IgG negative patients.

intraplaque neutrophil infiltration could be also explained by the increased release of neutrophilic chemoattractants (CXCL8 and TNF-α) from infiltrated macrophages (as suggested by our *in vitro* experiments with human macrophages incubated in an inflammatory microenvironment mimicking atherosclerotic plaque).

To summarize, our human, murine, and *in vitro* results suggest that anti-ApoA-1 IgG might be associated with increased atherosclerotic plaque vulnerability through two main mechanisms: (i) the increased intraplaque release of chemoattractants and MMP-9 and consequent collagen degradation; (ii) the induction of neutrophil infiltration from the blood stream within atherosclerotic plaques.

Those results may have two important clinical impacts for the management of cardiovascular disease in humans. First, our data

suggest that anti-ApoA-1 IgG assessment could potentially represent an emerging surrogate circulating marker of carotid atherosclerotic plaque vulnerability. Given the actual difficulty to assess carotid atherosclerotic plaque vulnerability by currently unstandardized methods and resource demanding imaging modalities,<sup>23</sup> the possibility of developing a cheap, standardized, reproducible, easily available, risk-stratification tool based upon anti-ApoA-1 IgG assessment constitutes a promising approach to better assess carotid atherosclerosis vulnerability. Second, by demonstrating in humans and in mice a direct negative effect of anti-ApoA-1 IgG on atherosclerotic plaque stabilization, it is likely that anti-ApoA-1 IgG could be considered an active factor increasing cardiovascular vulnerability. Given the recent demonstration of passive immunization approach as an emergent



therapeutic modality to treat some atherosclerotic diseases in humans and mice, we can hypothesize that intravenous administration of immunoglobulin (IVIG) or other more specific-targeted antibodies-based therapies could neutralize the deleterious effect of anti-ApoA-1 IgG on atherosclerotic plaque stability.<sup>24</sup> This potential approach was recently tested *in vitro* on cardiomyocytes, showing that treatment with IVIG abrogated the chronotropic effects of anti-ApoA-1 IgG.<sup>6</sup>

The limitation of the herein study is the relative limited sample size of carotid human specimens ( $n = 102$ ) due to the application of very stringent exclusion criteria to avoid any potential confounders, related to potential inflammatory and autoimmune co-morbidities, as well as anti-inflammatory medications. Thus, it may be possible that little changes in intraplaque (inflammatory gene and MMP-8 mRNA expression) and systemic (CCL4, MMP-9) parameters failed to reach statistical significance. Nevertheless, this limitation was the trade-off to obtain a pure model of human atherosclerosis in primary prevention, which also constitutes strength of this cohort. Whether our results apply also to unselected patients with carotid atherosclerosis remain to be demonstrated. Also, anti-ApoA-1 IgG positive differed from negative patients only for total serum cholesterol and triglyceride levels (both increased in anti-ApoA-1 IgG-negative patients). Given the role of circulating lipids as a well-known cardiovascular risk factor, this aspect suggests anti-ApoA-1 IgG-negative patients to potentially be at increased cardiovascular risk when compared with positive patients. However, as levels of total cholesterol and triglycerides were not markedly elevated in both groups, we can assume that these alterations in lipid profile did not reasonably induce any remarkable pro-atherosclerotic effects in the study population.<sup>25</sup> Finally, it must be reminded that there is currently no strict consensus about the exact definition of vulnerable plaque in humans, and about the model system that is best suited to investigate plaque rupture in mice.<sup>26</sup> The situation is further complicated by the fact that vulnerability criteria used in humans (mostly histological) might not apply to mice.<sup>23</sup> In this context, observing such a similarity between human and mice results undoubtedly constitutes another strength of the herein study. On the other hand, being focused on assessing the relevance of anti-ApoA-1 IgG in humans *in vitro*, we performed our *in vitro* experiments only on human primary immunocompetent cells, and not mice-derived neutrophils or macrophages. Therefore, we cannot formally extrapolate our *in vitro* results obtained on human cells to mice-derived inflammatory cells. This approach limited the direct relevance of anti-ApoA-1 IgG pro-inflammatory activities to humans instead of the animal model. Another limitation resides in the fact that we did not explore the potential interference of anti-ApoA-1 IgG with more classical ApoA-1-related properties, such as reverse cholesterol transport, anti-inflammatory, or anti-oxidant activities, which in turn might contribute to cardiovascular vulnerability.<sup>27</sup>

In conclusion, anti-ApoA-1 IgG increased cardiovascular vulnerability in humans and ApoE<sup>-/-</sup> mice. Intraplaque markers of vulnerability (such as MMPs, macrophage, and neutrophil content) were increased in anti-ApoA-1 IgG-positive patients and by anti-ApoA-1 IgG treatment in mice. Accordingly, anti-ApoA-1 IgG was inversely correlated with intraplaque collagen content.

Furthermore, *in vitro* anti-ApoA-1 IgG increased neutrophil migration in response to classical neutrophilic chemoattractants, expressed in atherosclerotic plaques. In addition, anti-ApoA-1 IgG stimulation was associated with the promotion of the release of monocyte and neutrophil chemoattractants, as well as MMP-9 by human macrophage. These results suggest that anti-ApoA-1 IgG might be associated with atherosclerotic plaque vulnerability. This could explain the poor cardiovascular prognosis observed in anti-ApoA-1-positive patients with MI or RA,<sup>5,6</sup> but remains to be demonstrated in larger prospective clinical studies.

## Supplementary material

Supplementary material is available at *European Heart Journal* online.

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